CHAPTER 4

FINDINGS AND RESULTS

4.1 Screening of biological activity of crude extracts

The ethanolic extracts of fruits of *Piper chaba* Linn, roots of *Piper sarmentosum* Roxb, stems of *Piper interruptum* Opiz, roots of *Plumbago indica* Linn and rhizomes of *Zingiber officinale* Roscoe were prepared as described in section 3.3. Percentage of yields of the extracts are shown in Table 4.1.

Table 4.1 %Yields of the ethanolic extracts from the investigated spices.

Plant Species	Extracts	Code	% Yield (w/w)
Piper chaba Linn.	EtOH	PC	10.89
Piper sarmentosum Roxb.	EtOH	PS	6.21
Piper interruptum Opiz.	EtOH	PI	2.47
Plumbago indica Linn.	EtOH	PL	10.61
Zingiber officinale Roscoe.	EtOH	ZO	4.30
Benjakul preparation	EtOH	BEN	11.10

4.1.1 Cytotoxic activity of Benjakul preparation

The cytotoxic activity of the ethanolic extracts of the five plants and Benjakul preparation were evaluated by the Sulphorhodamine B (SRB) assay. The results of cytotoxic activity of the extracts (screening) are shown in Tables 4.2.

The results of cytotoxicity evaluation of all plant extracts and Benjakul preparation at 50 µg/ml concentration and exposure time 72 hours are shown in Table 4-2. This data showed that the ethanolic extract of the fruit *Piper. chaba* exhibited high cytotoxic activity against COR-L23 and HepG2 cancer cell lines with the percentage of survival of 11.32 ± 1.64% and 18.11 ± 1.86%, respectively. The ethanolic extract of the stem of *P. interruptum* exhibited high cytotoxic activity against HepG2, Hela and COR-L23 cancer cell lines with the percentage of survival

of 1.34 ± 0.21%, 4.64 ± 0.09% and 8.59 ± 0.91%, respectively. The ethanolic extract of the root of *Plumbago indica* exhibited high cytotoxic activity against Hela, COR-L23 and HepG2 cancer cell lines with the percentage of survival of 1.51 ± 0.50%, 12.48 ± 3.55% and 13.70 ± 3.49%, respectively. The ethanolic extract of the rhizome of *Zingiber officinale* showed high cytotoxic activity against MCF-7 and COR-L23 with the percentage survival of 12.77 ± 1.81% and 17.43 ± 1.87%, respectively. The ethanolic extract of Benjakul preparation showed high cytotoxic activity only against COR-L23 where the percentage survival of the cancer cell line for 50 µg/ml concentration at exposure time 72 hours was 22.76 ± 2.49%. Among them, the ethanolic extract of the stem of *Piper. interruptum* contained the most cytotoxic effect against HepG2 (%survival = 1.34 ± 0.21) and COR-L23 (%survival = 8.59 ± 0.91) cancer cell lines, the ethanolic extract of the root of *Plumbago indica* exhibited the most cytotoxic effect against Hela (%survival = 1.51 ± 0.50) and the ethanolic extract of *Zingiber. officinale* possessed the most cytotoxic against MCF-7 cell line (%survival = 12.77 ± 1.81).

Table 4.2 Percentage of survival cells (Mean ± SEM) of four types of cancer cell lines (cervical cancer cell line [Hela], liver cancer cell line [HepG2], breast cancer cell line [MCF-7], lung cancer cell line [COR-L23] treated with extract concentration 50 µg/ml at exposure time 72 hrs (n = 6)

Plant Species	**Part**	**Extracts**	**Codes**	**Cell lines**			
				Hela	**HepG2**	**MCF-7**	**COR-L23**
Piper chaba	Fruit	EtOH	PC	42.13 ± 3.96	18.11 ± 1.86	32.50 ± 3.91	11.32 ± 1.64
Piper sarmentosum	Root	EtOH	PS	63.23 ± 5.43	75.52 ± 4.65	77.87 ± 2.00	55.49 ± 0.77
Piper interruptum	Stem	EtOH	PI	4.64 ± 0.09	1.34 ± 0.21	58.01 ± 6.11	8.59 ± 0.91
Plumbago indica	Root	EtOH	PL	1.51 ± 0.50	13.70 ± 3.49	35.84 ± 8.67	12.48 ± 3.55
Zingiber officinale	Rhizome	EtOH	ZO	25.44 ± 2.55	54.17 ± 4.03	12.77 ± 1.81	17.43 ± 1.87
Benjakul preparation	-	EtOH	BEN	32.41 ± 1.87	47.98 ± 4.99	53.83 ± 1.41	22.76 ± 2.49

n = number of independent experiment which was performed in 6 replicates.

Table 4.3 Cytotoxic activity (IC_{50} µg/ml ± SEM) of plant extracts against four types of cancer cell lines (Hela, HepG2, MCF-7 and COR-L23) and one type of normal cell line (MRC5) at exposure time 72 hrs (n=3)

Plant Species	Part	Extracts	Codes	Cell lines				
				Hela	**HepG2**	**MCF-7**	**COR-L23**	**MRC-5**
Piper chaba	Fruit	EtOH	PC	47.72 ± 2.11*	34.54 ±0.20**	35.17 ± 1.91*	15.82 ± 0.80*	91.71 ± 0.50
Piper sarmentosum	Root	EtOH	PS	69.35 ± 3.36*	72.27 ± 0.90*	69.53 ± 9.09*	32.91 ± 1.71*	>100
Piper interruptum	Stem	EtOH	PI	28.65 ± 1.10**	26.12 ± 0.65*	62.35 ± 5.23*	18.40 ± 0.61*	34.44 ± 1.61
Plumbago indica	Root	EtOH	PL	8.71 ± 0.40*	33.22 ± 0.24*	40.81 ± 3.62*	3.43 ± 1.93*	70.04 ± 2.16
Zingiber officinale	Rhizome	EtOH	ZO	37.29 ± 0.23*	51.63 ± 0.31*	31.15 ± 1.40*	7.90 ± 1.62*	83.45 ± 5.37
Benjakul preparation	-	EtOH	BEN	47.72 ± 2.11	45.58 ± 1.26	33.20 ± 0.80*	19.80 ± 1.89*	48.95 ± 0.34

The comparison of cytotoxic activity against cancer cell and normal cell showed significantly by * $p<0.5$, ** $p<0.05$. n = number of independent experiment which was performed in 3 replicates.

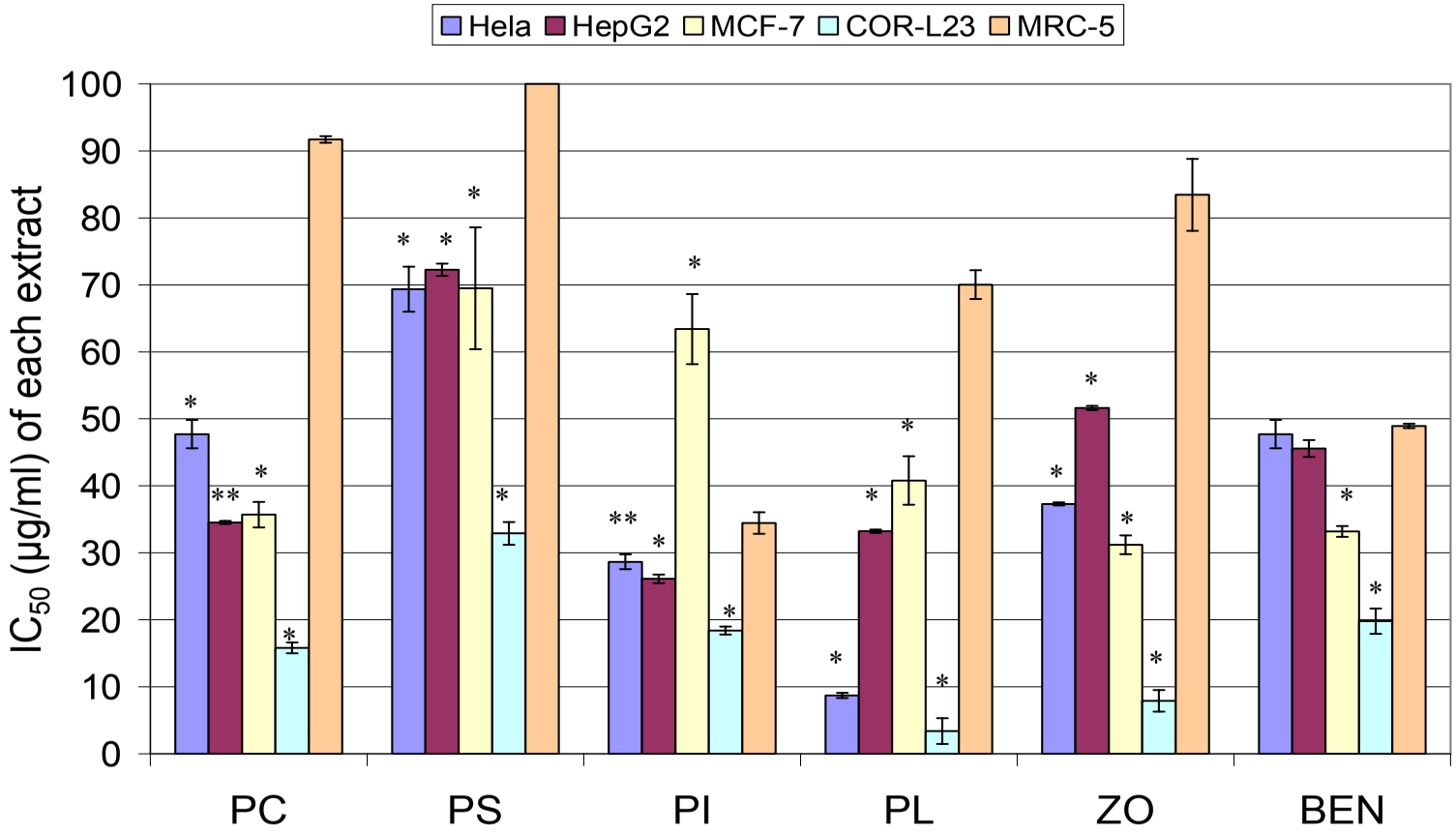


Figure 4.1 Cytotoxic activity [IC_{50} (µg/ml)] of five plants and Benjakul preparation on five types of cell lines exposure time 72 hr (n=3) using student t-test from prism to compare the significant difference between normal cell (MRC-5) and each cancer cell (Hela, HepG2, MCF-7 and COR-L23) (* $p<0.05$, **$p<0.0001$).

Calculations of the IC_{50} values of all plants are shown in Table 4.3 and Figure 4.1. This data showed that the ethanolic extracts of four plants and Benjakul preparation showed cytotoxic activity against COR-L23 and one plant showed cytotoxic activity against Hela followed by the American National Cancer Institue (NCI) (IC_{50} <20 μg/ml for crude extract) (Boyde,1997). The data showed that the IC_{50} value of the ethanolic extracts of the root of *Plumbago indica* showed the highest cytotoxic activity against COR-L23 and the second most effective activity against Hela. The IC_{50} value of the ethanolic extract of the root of *P. indica* was 3.43 ± 1.93 and 8.71 ± 0.40 μg/ml, respectively. The ethanolic extract of the fruit of *Piper chaba*, the stem of *Piper interruptum*, the rhizome of *Zingiber officinale* and Benjakul preparation showed the highest cytotoxic activity against COR-L23 with the IC_{50} value of 15.82 ± 0.80, 18.40 ± 0.61, 7.90 ± 1.62 and 19.80 ± 1.89 μg/ml, respectively. All extracts exhibited specific activity against COR-L23 higher than Hela, HepG2 and MCF7 but less active with normal cell line (MRC-5) with P-value was significant ($p<0.05$) calculated by student t-test from Prism program. From this result, it was concluded that the ethanolic extract of the five plants and Benjakul preparation showed cytotoxicity against COR-L23 and deserved for the plant extracts or their active ingredients which can kill cancer cells but less harmful to normal cells. Thus, these results related with the objectives of cancer chemotherapy which can kill cancer cells with as little damage as possible to normal cells and should be selectively active (Halliwell & Gatteridge, 1988).

Thus, isolation of pure compounds from the ethanolic extracts of Benjakul preparation was continued for investigation because it showed high cytotoxic activity against lung cancer cell line but less activity against lung normal cell line.

4.2 Cytotoxic activity of bioassay-guided fractionation

Results from the preliminary assays for cytotoxic activity of the ethanolic extracts of five plants and Benjakul preparation are shown in section 4.1. They give evidences of the presence of active constituents in the ethanolic extracts of Benjakul preparation, so the separation of these active extracts was carried out by bioassay

guide fractionation described in section 3.5. Five fractions from the ethanolic extracts of Benjakul preparation (BEN1, BEN2, BEN3, BEN4 and BEN5) were tested for cytotoxic activity against COR-L23 using the SRB method (data shown in Table 4.4 and Figure 4.2).

Table 4.4 IC_{50} (µg/ml) ± SEM of the fractions from Benjakul preparation separated by vacuum liquid chromatography against COR-L23 at exposure time 72 hours (n=3)

Fraction	%Yield	IC_{50} ± SEM (µg/ml)
		COR-L23
BEN1 (Hexane)	0.54	50.12 ± 1.54
BEN 2 (Hexane:$CHCl_3$)	1.15	20.55 ± 2.25
BEN 3 ($CHCl_3$)	14.03	7.38 ± 0.21
BEN 4 ($CHCl_3$:MeOH)	54.30	62.81 ± 2.02
BEN 5 (MeOH)	15.65	>100

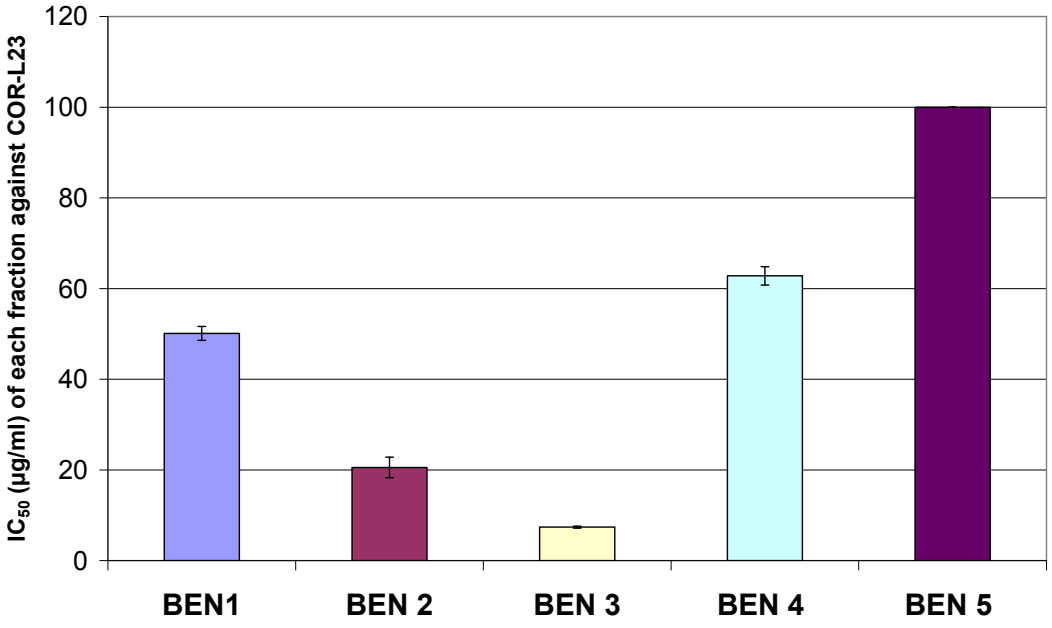


Figure 4.2 Histogram comparing IC_{50} of each fraction against COR-L23 at exposure time 72 h.

BEN1, BEN2 BEN4 and BEN5 showed less cytotoxicity against COR-L23 than the crude ethanolic extract of Benjakul preparation (Table 4.3). BEN3 was the only one fraction which showed high and specific cytotoxicity against COR-L23. From this result, fraction BEN3 was explicitly chosen for separation to find active compounds because of its high and specific cytotoxicity.

4.3 Analysis of isolated compounds and structure elucidation

4.3.1 Structure elucidation of the isolated compounds

Results from the bioassay-guided fractionation for cytotoxic activity were shown in section 4.2. Thus, a separation of the active extracts was carried out as shown in section 3.6 to give the pure compounds as follows.

4.3.1.1 BENS1

BENS1 (Piperine): $C_{17}H_{19}NO_3$ (158.5 mg, 7.81%w/w); yellow pale needle crystals; EIMS (low resolution) *m/z* (% relative intensity) 285 (M^+, 75), 201 (100), 173 (19), 143 (17), 115 (45). BENS1 was the major compound isolated from the ethanolic extract of Benjakul preparation, obtained as yellow pale needle crystals. The ^{1}H-NMR and EIMS spectrums are shown in Table 4.5, Figure 4.4 and Figure 4.5. The TLC analysis of this compound was compared with authentic sample piperine (Merck) by TLC using 3 solvent systems and gave identical behavior. The ^{1}H NMR spectrum, compared with the previous ^{1}H-NMR data of piperine, was the same as the spectrum recorded for piperine (Araujo-Junior *et al.,* 1997). Thus, it was strongly supported that BENS1 to be piperine. The structure was shown in Figure 4.3.

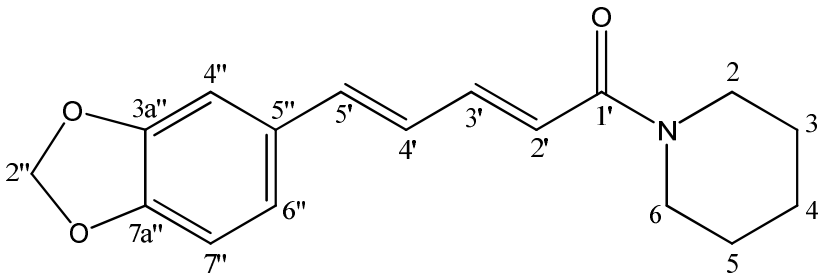


Figure 4.3 Structure of piperine

Table 4.5 ^{1}H-NMR spectral data (500 MHz) of BENS1 in $CDCl_3$ and CD_3OD

Position	δ_H (mult., *J* in Hz) of Piperne	δ_H (mult., *J* in Hz) of BENS1
2	3.48 (*br.s*; 2H)	3.64 (*dd*; 2H, 11.1, 5.7)
3	1.49 (*m*; 2H)	1.62 (*m*; 2H)
4	1.56 (*m*; 2H)	1.71 (*m*; 2H)
5	1.49 (*m*; 2H)	1.62 (*m*; 2H)
6	3.48 (*br.s*; 2H)	3.64 (*dd*; 2H, 11.1, 5.7)
2′	6.36 (*d*, 14.6)	6.65 (*d*, 14.7)
3′	7.31 (*m*)	7.34 (*dd*, 14.7, 9.6)
4′	6.64 (*m*)	6.88 (*dd*, 14.7, 9.9)
5′	6.65 (*m*)	6.89 (*d*, 14.7)
2″	5.86 (*s*; 2H)	5.98 (*s*; 2H)
4″	6.88 (*d*, 1.6)	7.11 (*d*, 1.5)
6″	6.79 (*dd*, 1.6, 8.0)	6.98 (*dd*, 8.1, 1.5)
7″	6.67 (*d*, 8.0)	6.81 (*d*, 8.1)

Note: Piperine from Araujo-Junior *et al.,* 1997

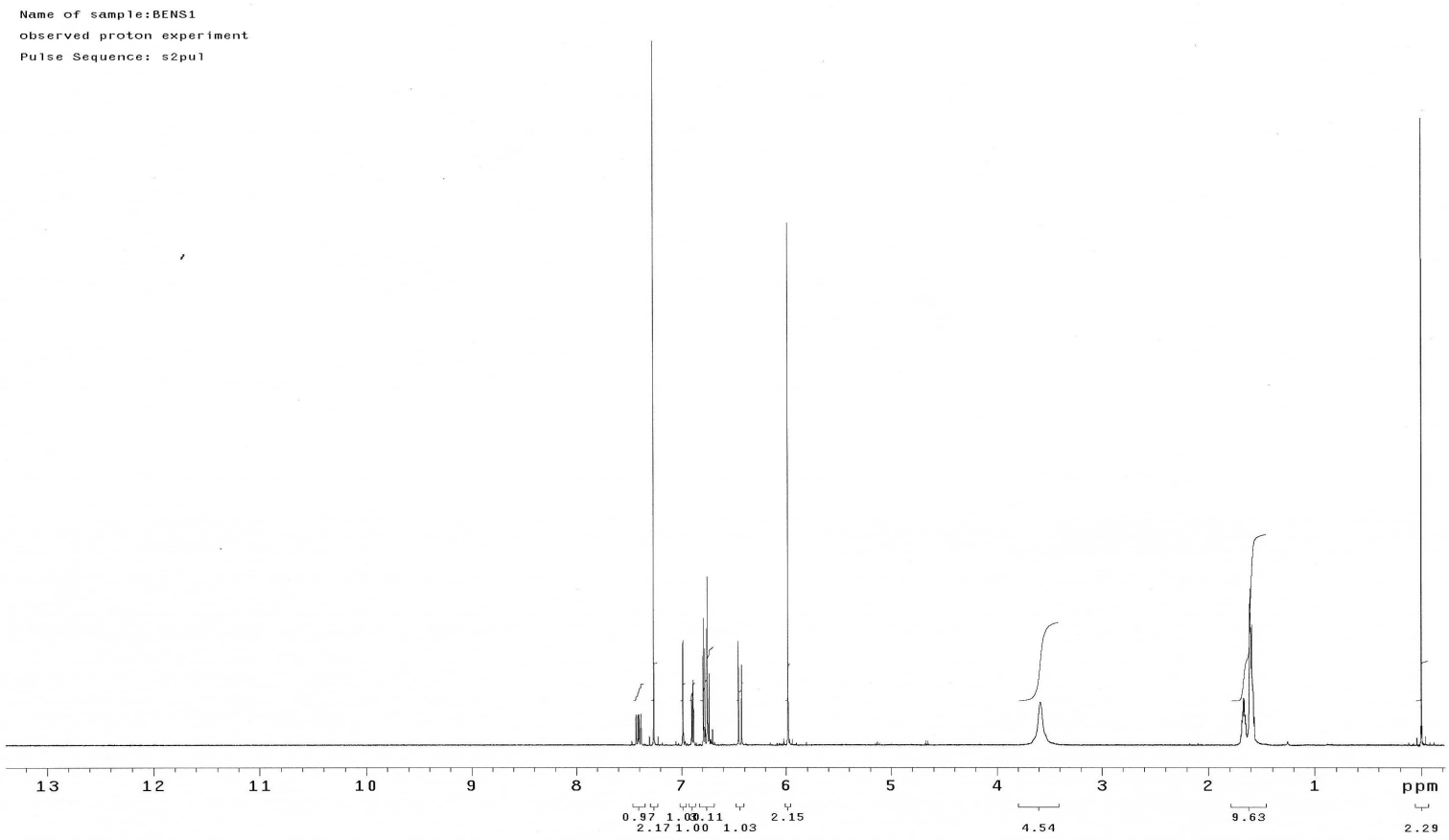


Figure 4.4 ^{1}H NMR spectrum of BENS1 in $CDCl_3$ and CD_3OD

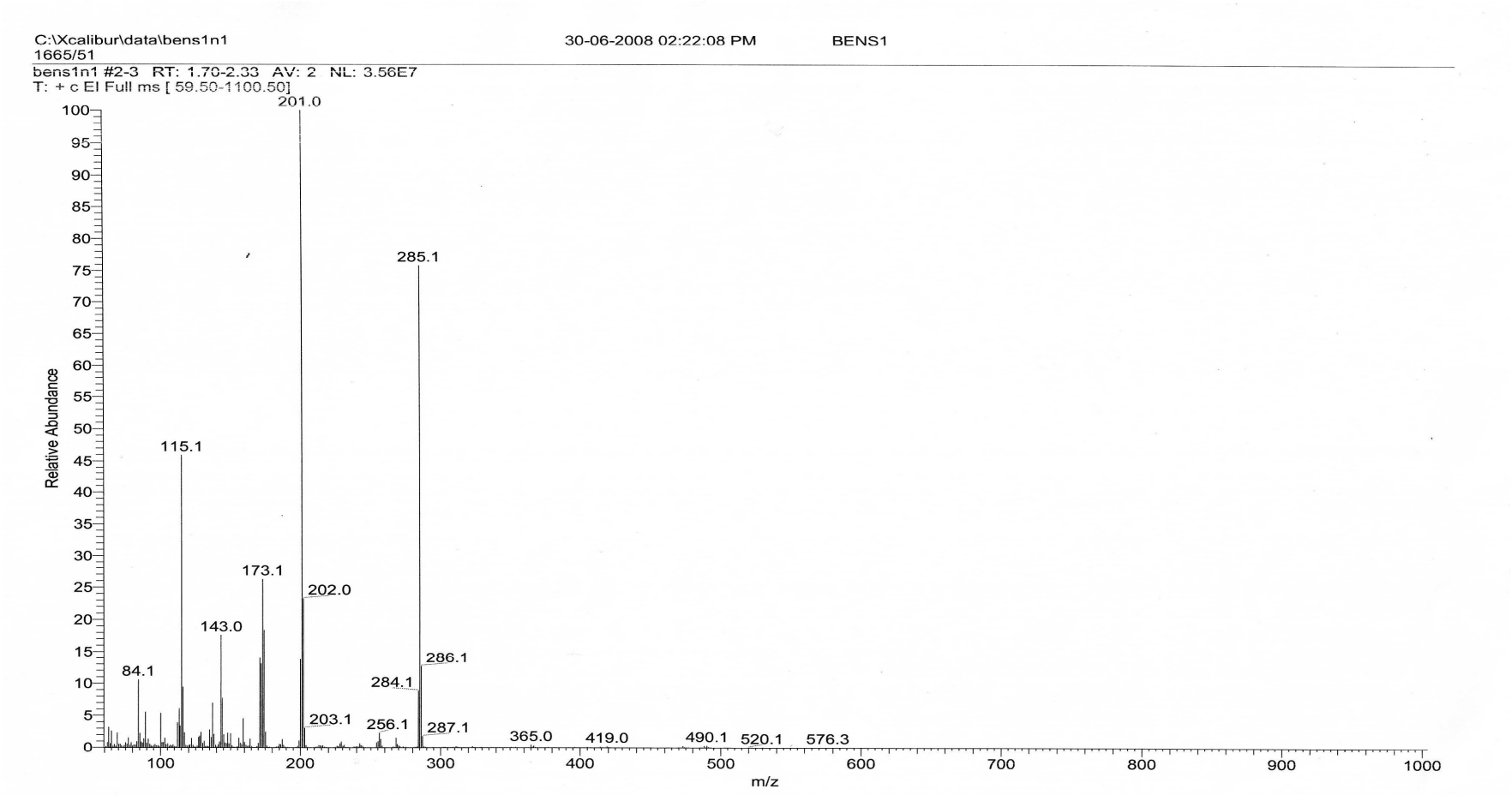


Figure 4.5 EIMS spectrum of BENS1

4.3.1.2 BENS2

BENS2 (Plumbagin): $C_{11}H_8O_3$ (74.9 mg, 4.18%w/w); orange needle crystals; EIMS (low resolution) *m/z* (% relative intensity) 188 (M^+, 100), 131 (54), 81 (62), 69 (98). BENS2 was the compound isolated from the ethanolic extract of Benjakul preparation, obtained as orange needle crystals. The ^{1}H-NMR and EIMS spectrums are shown in Table 4.6, Figure 4.7 and Figure 4.8. The TLC analysis of this compound was compared with authentic sample plumbagin (Sigma) by TLC using 3 solvent systems and gave identical behavior. The ^{1}H-NMR spectrum, compared with the previous ^{1}H-NMR data of plumbagin, was the same as the spectrum recorded for plumbagin (Nahálka *et. al.,* 1996). Thus, it was strongly supported that BENS2 to be plumbagin. The structure was shown in Figure 4.6.

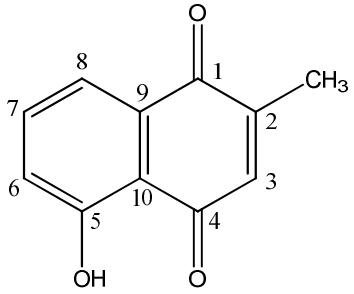


Figure 4.6 Structure of plumbagin

Table 4.6 ^{1}H-NMR spectral data (500 MHz) of BENS2 in $CDCl_3$ and CD_3OD

Position	δ_H (mult., *J* in Hz) of Plumbagin	Position	δ_H (mult., *J* in Hz) of BENS2
2	2.15 (*d*; 3H, 1.57)	2	2.17 (*s*; 3H)
3	6.73 (*q*, 1.58)	3	6.88 (*q*, 1.5)
6-8	7.1–7.6 (*m*)	6	7.27 (*dd*, 8.1, 1.2)
		7	7.68 (*t*, 8.1)
		8	7.61 (*dd*, 8.1, 1.2)
10-OH	10.8 (*s*)	10-OH	-

Note: Plumbagin from Nahálka *et al.,* 1996

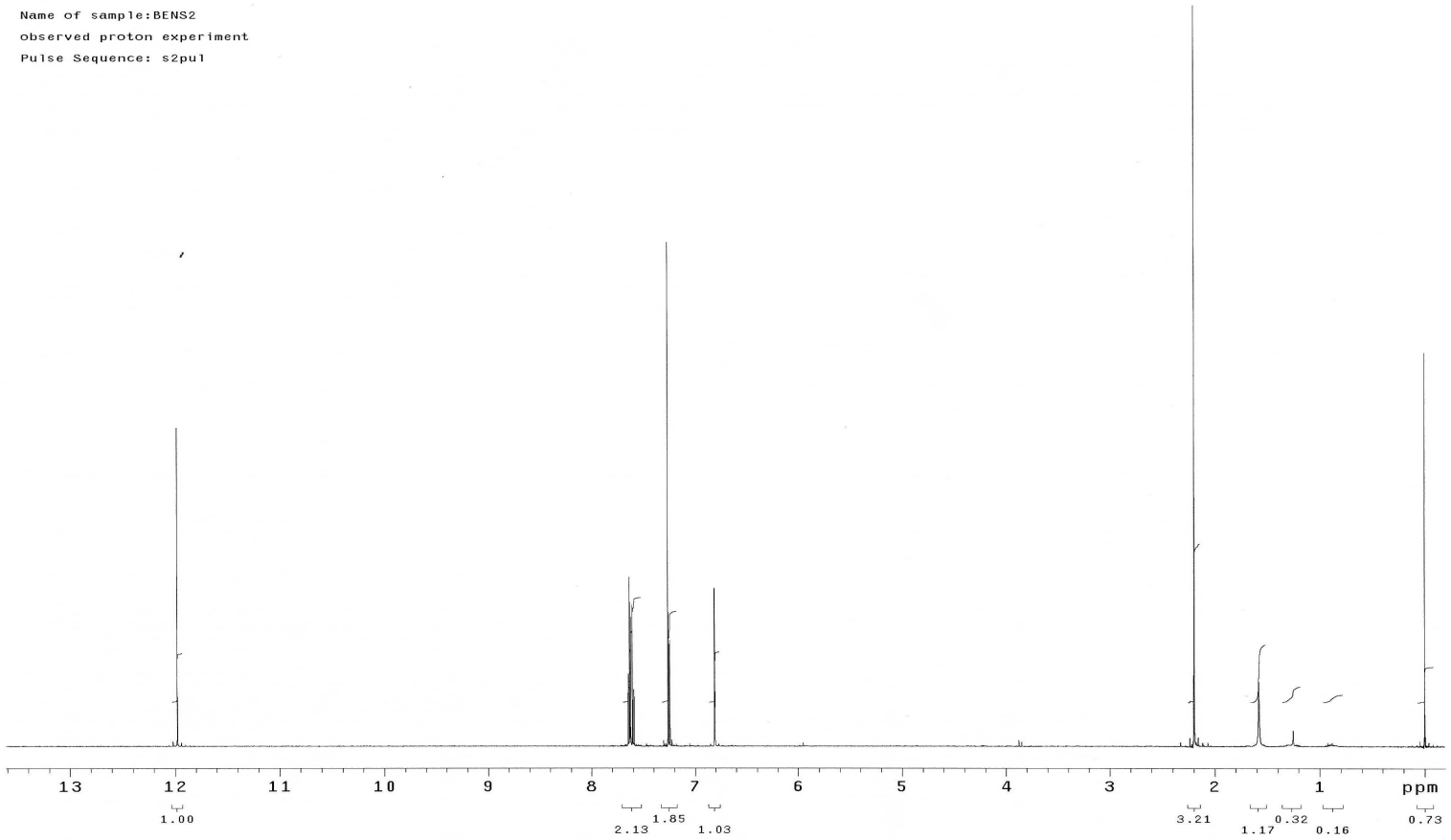


Figure 4.7 ^{1}H NMR spectrum of BENS2 in $CDCl_3$ and CD_3OD

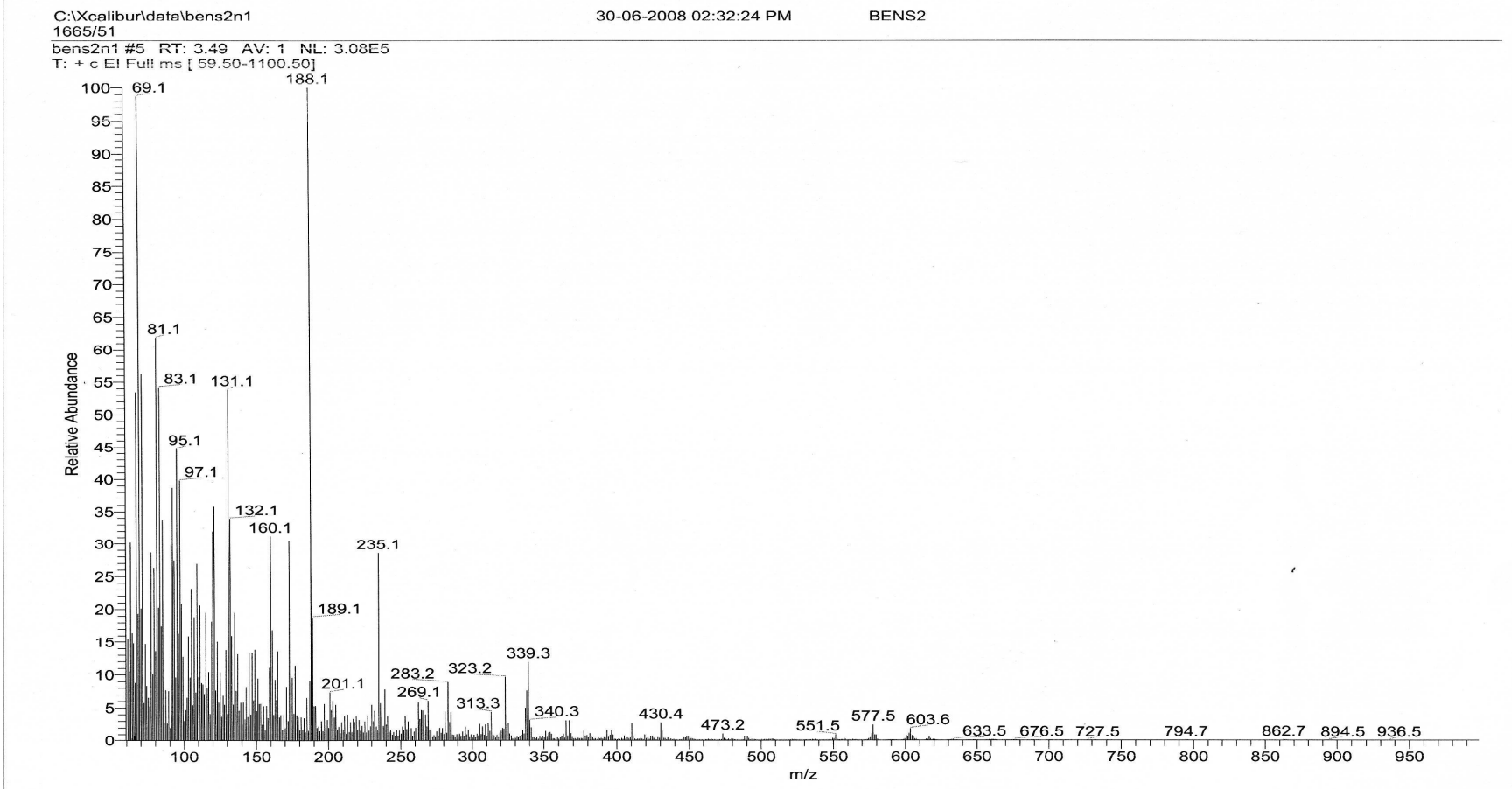


Figure 4.8 EIMS spectrum of BENS2

4.3.1.3 BENS3

BENS3 (6-gingerol): $C_{17}H_{26}O_4$ (9.6 mg, 0.54%w/w); yellow pale oil; EIMS (low resolution) *m/z* (% relative intensity) 294 (M^+, 50), 150 (55), 137 (100). BENS3 was the compound isolated from the ethanolic extract of Benjakul preparation, obtained as yellow pale oil. The ^{1}H-NMR spectrums are shown in Table 4.7, Figure 4.10 and Figure 4.11. The TLC analysis of this compound was compared with authentic sample 6-gingerol (Wako) by TLC using 3 solvent systems and gave identical behavior. The ^{1}H NMR spectrum, compared with the previous ^{1}H-NMR data of 6-gingerol, was the same as the spectrum recorded for 6-gingerol (Kim *et al.,* 2008). Thus, it was strongly supported that BENS3 to be BENS3. The structure was shown in Figure 4.9.

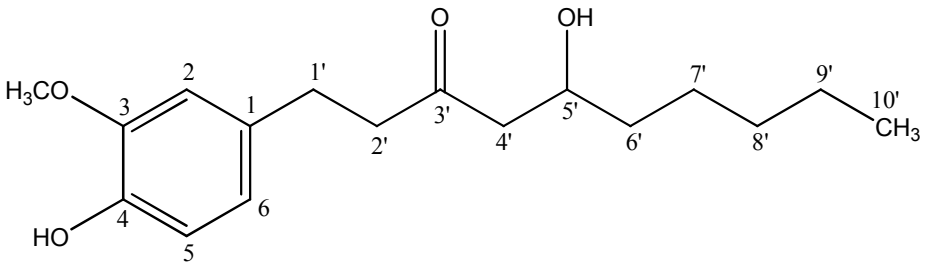


Figure 4.9 Structure of 6-gingerol

Table 4.7 ^{1}H-NMR spectral data (500 MHz) of BENS3 in $CDCl_3$

Position	δ_H (mult., *J* in Hz) of 6-Gingerol	Position	δ_H (mult., *J* in Hz) of BENS3
2	6.69 (*d*, 2.0)	2	6.68 (*br.s*)
3-OCH_3	3.68 (*s*; 3H)	3-OCH_3	3.87 (*s*; 3H)
4-OH	-	4-OH	5.51 (*s*)
5	6.60 (*d*, 8.4)	5	6.82 (*d*, 8.4)
6	6.53 (*dd*, 8.4, 2.0)	6	6.65 (*dd*, 8.4, 2.1)
1′-2′	2.69 (*s*; 4H)	1′	2.74 (*br.d*,2H 7.2)
		2′	2.71 (*dd*, 6.6, 2.1)
			2.85 (*dd*, 6.6, 2.1)
4′	2.44 (*dd*; 2H, 8.4, 2.0)	4′	2.48 (*dd*, 17.4, 3.3)
			2.58 (*dd*, 17.4, 8.7)
5′	3.86 (*m*)	5′	4.04 (*m*)
6′-9′	1.21–1.32 (*m*; 8H)	6′-9′	1.25–1.50 (*m*; 8H)
10′	0.83 (*t*; 3H)	10′	0.89 (*t*; 3H, 6.6)

Note: 6-gingerol from Kim *et al.,* 2008

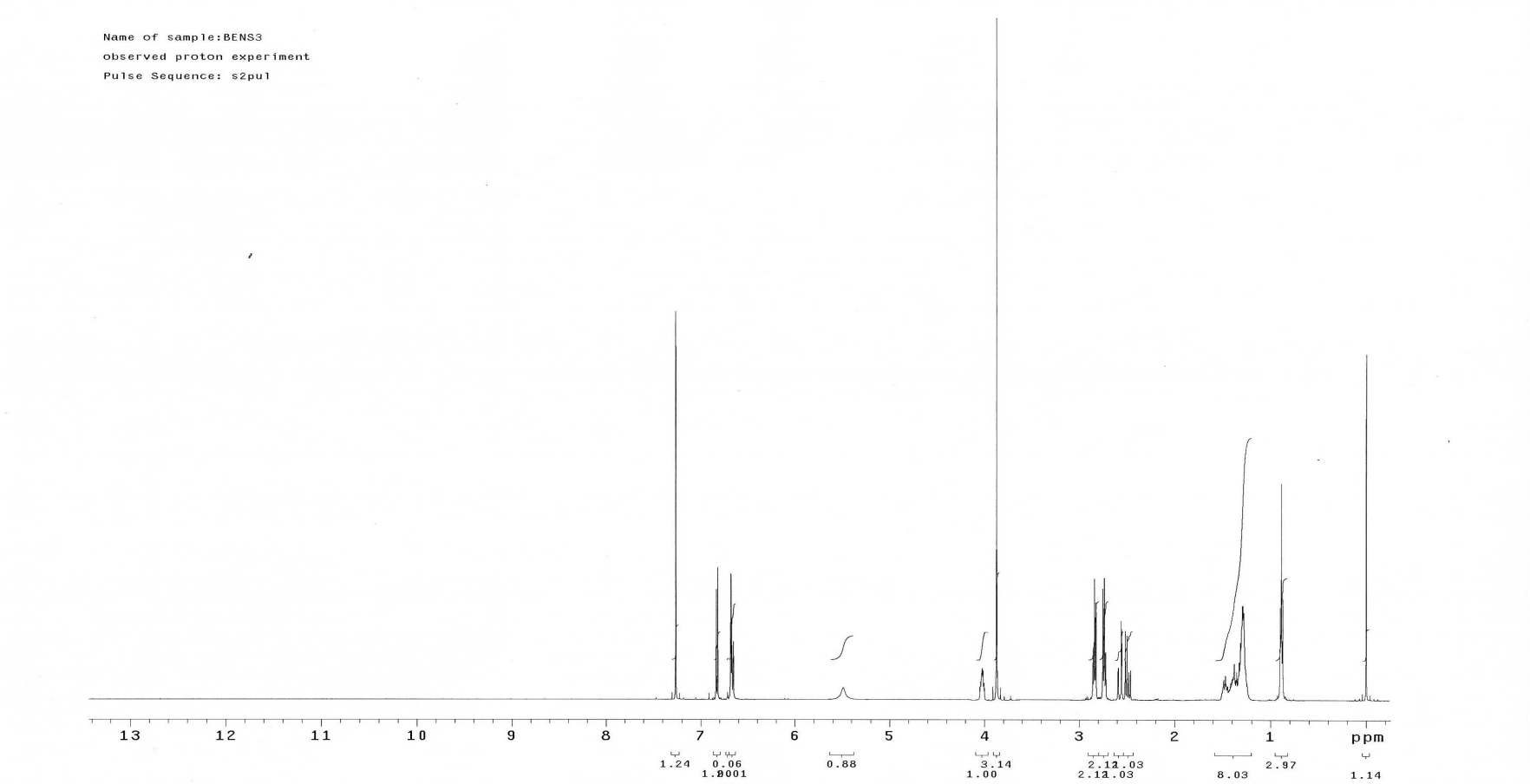


Figure 4.10 ^{1}H NMR spectrum of BENS3 in $CDCl_3$

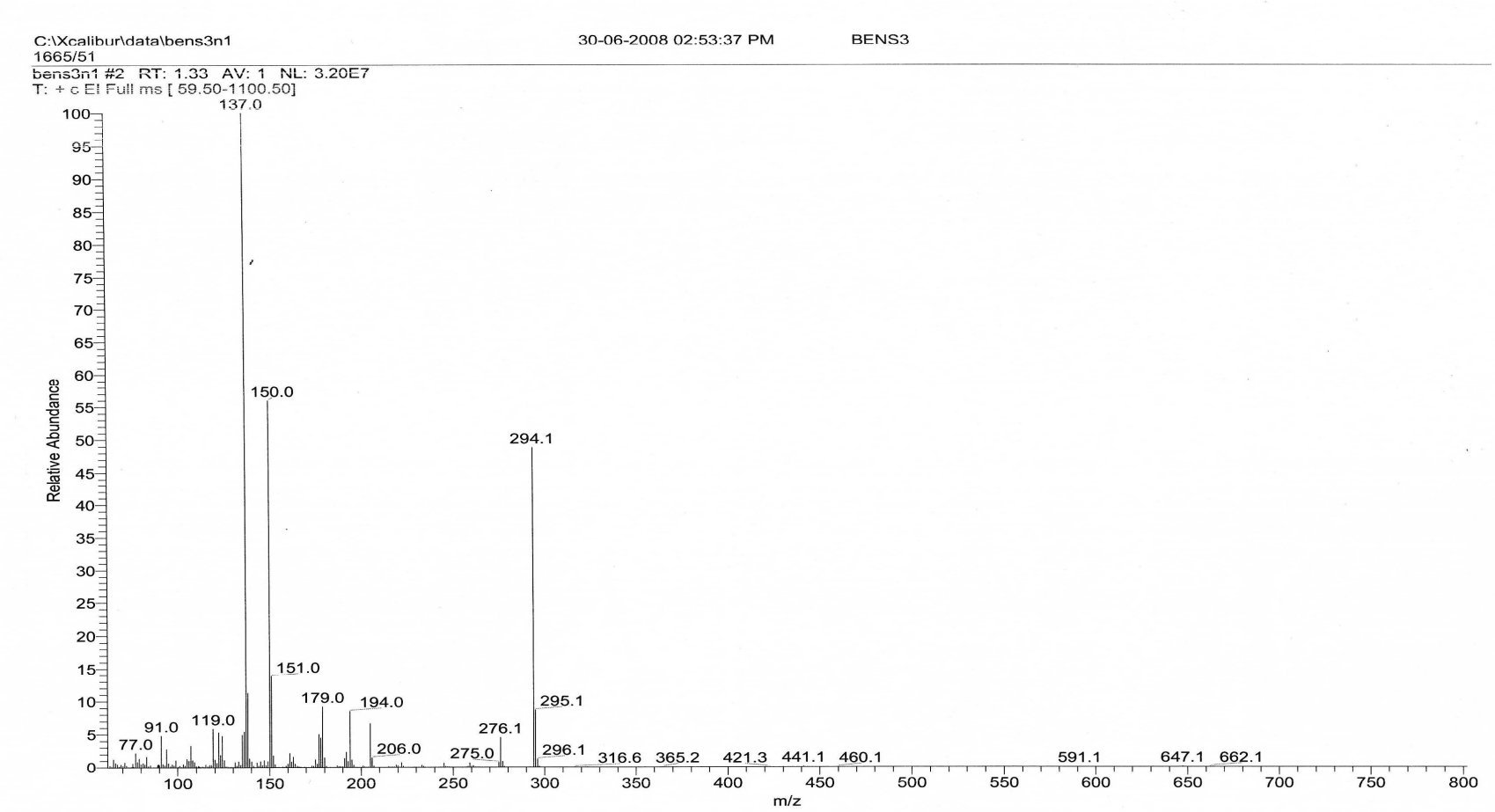
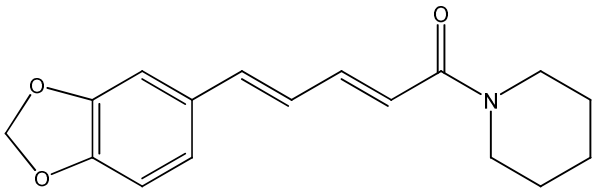


Figure 4.11 EIMS spectrum of BENS3

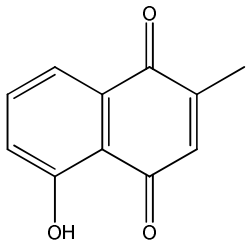
4.4 Discussion on phytochemical investigation

The ethanolic extract of Benjakul preparation was separated by column chromatography and used a solvent gradient of hexane, chloroform and methanol. The three compounds were isolated in this research. All pure compounds were detected by use of the general spraying reagent anisaldehyde in sulphuric acid. BENS1 (brown colour in daylight) showed a brown colour, BENS2 (yellow colour in daylight) showed a yellow brown colour and BENS3 showed a brown colour. Only two compounds (BENS1 and BENS 2) could be detected by UV 254 nm.

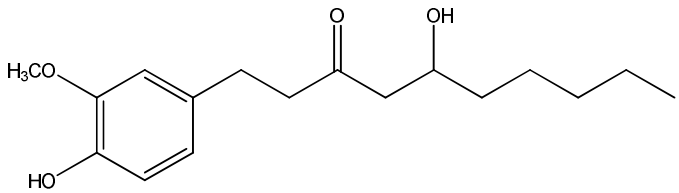
The three compounds from ethanolic extract of Benjakul preparation could be divided into three chemical groups. They were piperidine (BENS1 or piperine), naphthoquinone (BENS2 or plumbagin) and gingerol-related compound (BENS3 or 6-gingerol). The structures are shown in Figure 4.12. The investigation on chemical constituents of Benjakul preparation found that piperine, which is the main compound, was normally found in *Piper* species such as *Piper nigrum* and *Piper chaba* (Wu *et. al.,* 2004; Park *et. al.,* 2007). Plumbagin was found in *Plumbago* species such as *Plumbago zeylanica* and *Plumbago indica* (Dinda & Chel, 1992; Wang & Huang, 2005) 6-gingerol was found in *Zingiber* species such as *Zingiber officinale* (Surh *et. al.,* 1999; Wei *et. al.,* 2005).



BENS1 or Piperine



BENS2 or Plumbagin



BENS3 or 6-gingerol

Figure 4.12 The chemical structure of three compounds isolated from the ethanolic extract of Benjakul preparation.

4.5 Activities of the isolated compounds

The three isolated compounds (piperine, plumbagin and 6-gingerol) were assessed for cytotoxic activity against the four cancer cell lines (Hela, HepG2, MCF-7 and COR-L23) and one type normal cell lines (MRC5).

4.5.1 Cytotoxic activity of the isolated compounds

The results of pure compounds which were isolated from the ethanolic extract of Benjakul preparation against cervical cancer cell line (Hela), liver cancer cell line (HepG2), breast cancer cell line (MCF-7), lung cancer cell line (COR-L23) and normal lung fibroblast cell line (MRC5) at exposure time 72 hours are shown on Table 4.8 and Figure 4.13.

Following the criteria for cytotoxic activity of pure compounds established by the American National Cancer Institute (NCI) should show the IC_{50} less than 4 μg/ml. Among these compounds, piperine (IC_{50} 23.12 μg/ml or 81.12 μM) and 6-gingerol (29.12 μg/ml or 99.05 μM) showed less cytotoxic activity against cervical cancer cell (Hela), whereas plumbagin (IC_{50} 0.78 μg/ml or 4.15 μM) exhibited high potency. The effect of the compounds against liver cancer cells (HepG2) found that piperine (IC_{50} 17.56 μg/ml or 61.61 μM) and 6-gingerol (IC_{50} 14.69 μg/ml or 49.97 μM) also showed less activity but plumbagin (IC_{50} 0.49 μg/ml or 2.61 μM) showed the highest activity against HepG2. The result on breast cancer cells (MCF-7), piperine (IC_{50} 10.18 μg/ml or 35.72 μM) and 6-gingerol (IC_{50} 9.80 μg/ml or 33.33 μM) showed slightly activity and plumbagin (IC_{50} 0.43 μg/ml or 2.29 μM) showed the highest activity. The effect of the compounds against lung cancer cells (COR-L23) found that 6-ginerol (IC_{50} 25.31 μg/ml or 86.09 μM) showed less activity, piperine (IC_{50} 12.38 μg/ml or 43.44 μM) showed slightly activity and plumbagin (IC_{50} 0.48 μg/ml or 2.55 μM) exhibited the highest activity.

Although plumbagin exhibited the highest cytotoxic activity against all types of cancer cell lines, but also showed cytotoxic activity against normal cell line (MRC5). It showed that plumbagin is nonspecific cytotoxic activity.

Table 4.8 Cytotoxic activity (IC_{50} µg/ml ± SEM and µM) of isolated compounds against four types of cancer cell lines (Hela, HepG2, MCF-7 and COR-L23) and one type of normal cell line (MRC5) at exposure time 72 hr (n=3)

Compounds	**IC_{50} (µg/ml ± SEM) [µM]**				
	Hela	**HepG2**	**MCF-7**	**COR-L23**	**MRC-5**
BENS1 (Piperine)	23.12 ± 1.53	17.56 ± 2.32	10.18 ± 1.29	12.38 ± 1.45	> 50
	[81.12]	[61.61)	[35.72]	[43.44]	[>175.44]
BENS2 (Plumbagin)	0.78 ± 0.06	0.49 ± 0.01	0.43 ± 0.02	0.48 ± 0.02	2.17 ± 0.77
	[4.15]	[2.61]	[2.29]	[2.55]	[11.54]
BENS3 (6-gingerol)	29.12 ± 2.30	14.69 ± 1.47	9.80 ± 2.37	25.31 ± 0.90	> 50
	[99.05]	[49.97]	[33.33]	[86.09]	[>170.07]

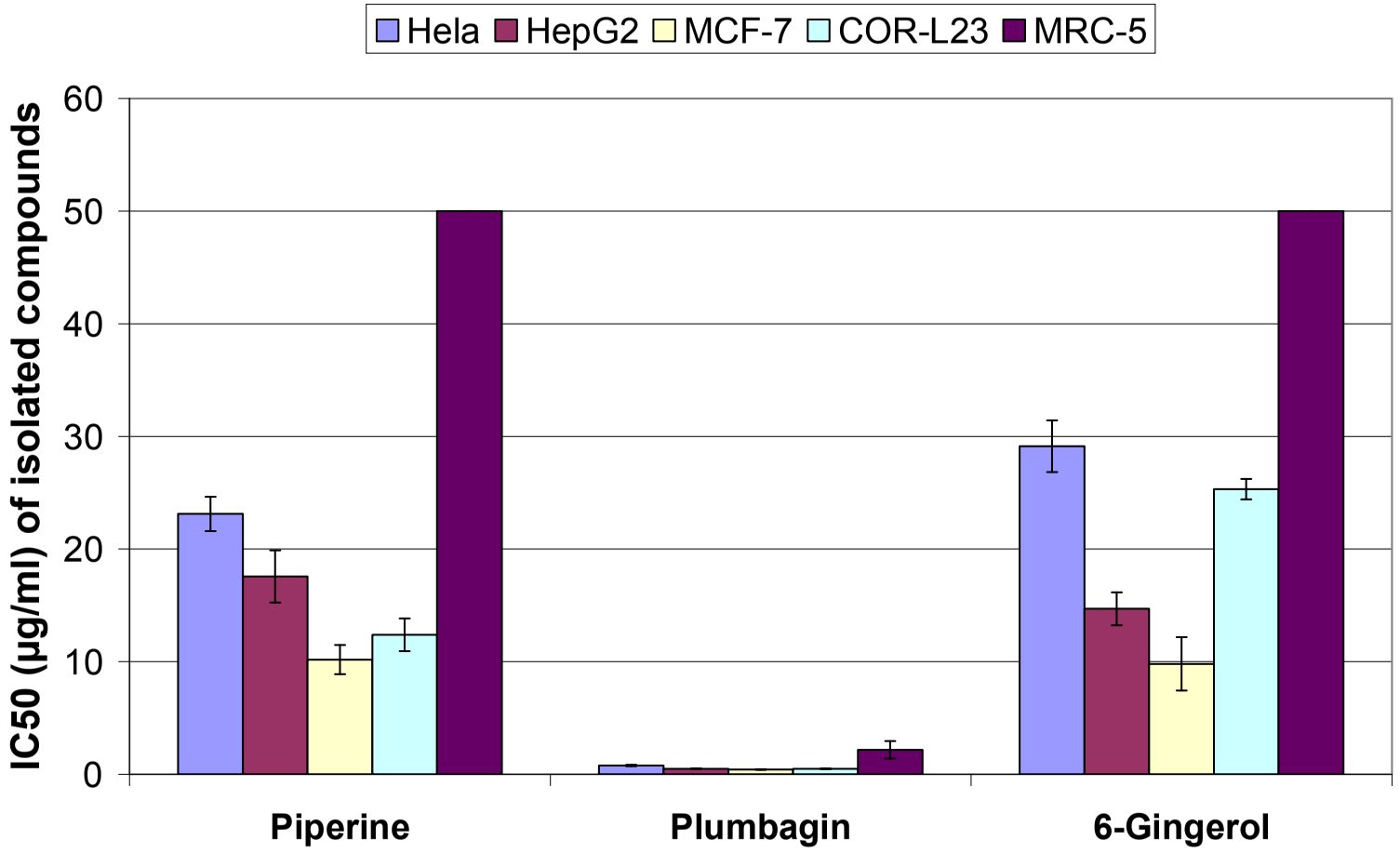


Figure 4.13 Histogram comparing IC_{50} values of isolated compounds of Benjakul preparation against four cancer cell lines (Hela, HepG2, MCF-7 and COR-L23) and one normal cell lines (MRC-5) at exposure time 72 hrs (n=3)

4.6 Study on chemical fingerprint of Benjakul preparation using high performance liquid chromatography

Results from the cytotoxic activity and analysis of active constituents of the ethanolic extracts of Benjakul preparation give evidences of the potential for production this preparation in manufacturing level for using in cancer patients. Piperine has been identified as main compound and plumbagin as the most cytotoxic compound, which can serve as a marker and these results were used for standardization of ethanolic extract of Benjakul preparation. High performance liquid chromatography (HPLC) method has been choose for investigation chemical fingerprint and quality control because this method is good sensitivity, precision, accuracy.

4.6.1 Development of chromatographic method

The ethanolic extract of Benjakul preparation was studied on chemical fingerprint by high performance liquid chromatography which is show in section 3.7. The liquid chromatographic conditions are summarized in Table 4.4. A representative chromatogram is shown in Figure 4.14.

Table 4.9 HPLC conditions for analysis of ethanolic extract of Benjakul preparation.

Operating parameters	Conditions
Stationary Phase	Phenomenex Luna 5µ C18(2) 100A (250 x 4.60 mm 5 micron)
Mobile Phase	water-acetronitrile with gradient elution as follows: 0 min, 60:40; 30 min, 50:50; 50 min, 5:95; 60 min, 0:100
Flow Rate	1.0 ml/min
Wavelength	256 nm
Injection Volume	10 µl

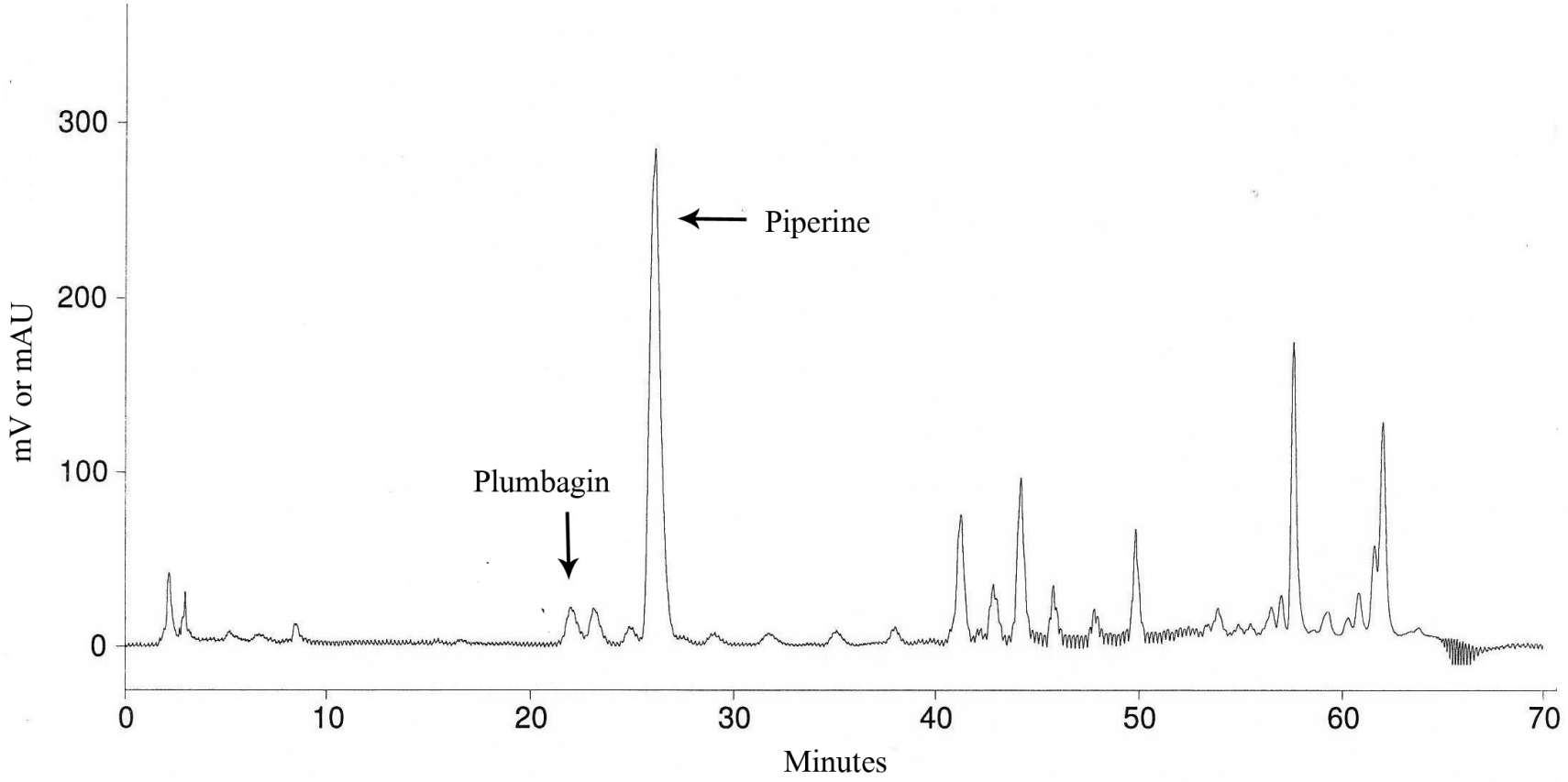


Figure 4.14 HPLC chromatogram of ethanolic extract of Benjakul preparation (10 mg/ml). Mobile phase: water-acetronitrile with gradient elution as follows: 0 min, 60:40; 30 min, 50:50; 50 min, 5:95; 60 min, 0:100; Flow rate 1.0 ml/min; UV detector at 256 nm.

Based on the structure of piperine and plumbagin, the compounds would be characterized by moderate-low polarity. Therefore, a C18 reversed phase HPLC column was choose for quantification of piperine and plumbagin. A moderately to highly polar mobile phase composed of water and acetronitrile with gradient elution as follows: 0 min, 60:40; 30 min, 50:50; 50 min, 5:95; 60 min, 0:100, was eluted with 1 ml/min flow rate. Detection wavelength was 256 nm bases on λ_{max} of plumbagin.

4.7 HPLC method validation

Chromatographic method that development as describe in section 4.6 was validated following in section 3.8.

4.7.1 Specificity validation

The results of HPLC chromatograms for specificity validation are shown in Figure 4.15. In Figure 4.15, it is apparent that piperine is a major compound of ethanolic extract of Benjakul preparation, with 28.04 min of retention time. Plumbagin is a minor compound of ethanolic extract of Benjakul preparation, with 24.59 min of retention time. But, there are interfering peaks observed around the peak of piperine and plumbagin.

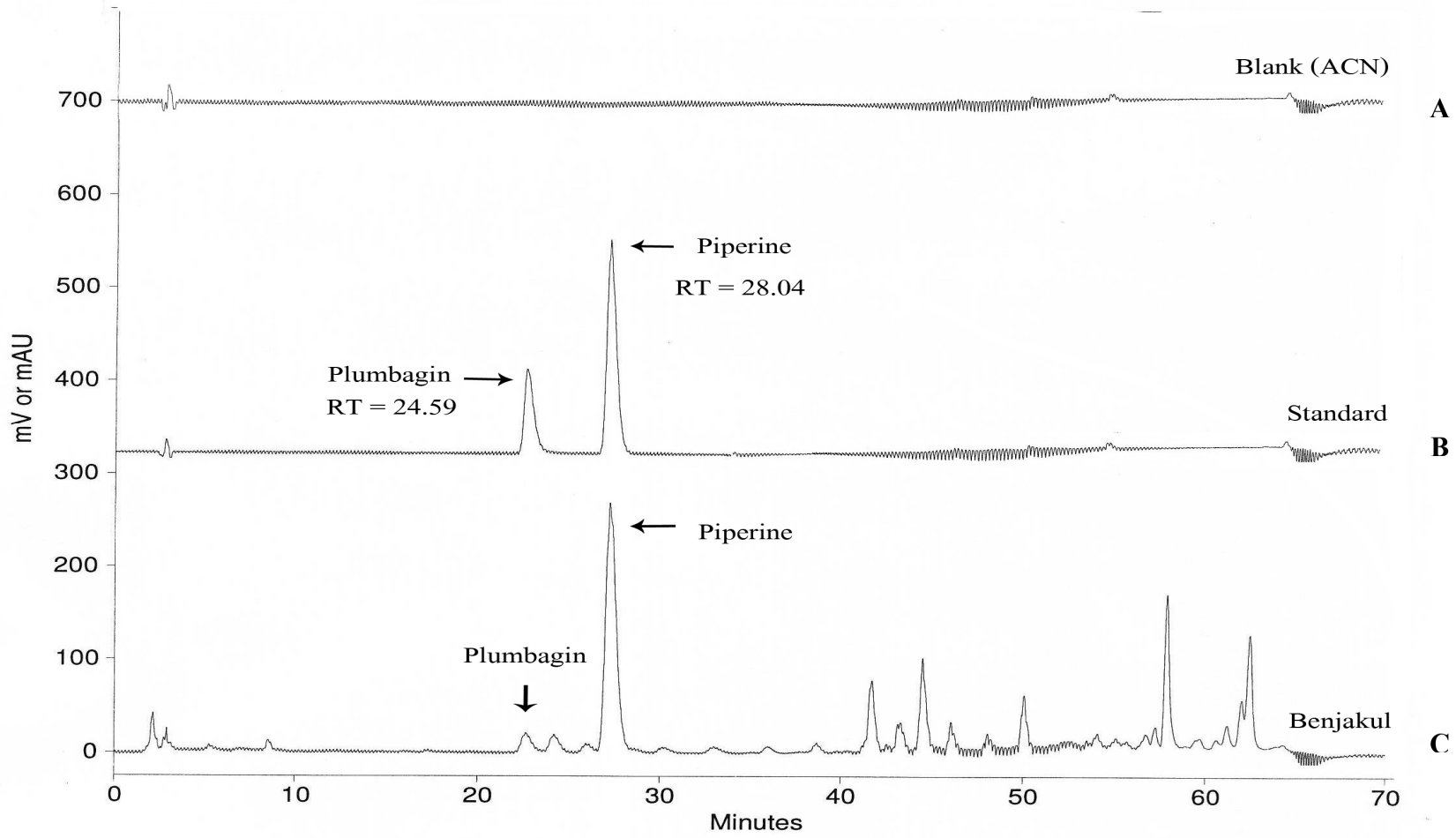


Figure 4.15 The specificity validation for the HPLC analytical method for piperine and plumbagin: (A) blank solution; (B) piperine and plumbagin standards solution and (C) ethanolic extract of Benjakul preparation sample solution.

4.7.2 Quantitation parameters

Serial dilutions of standard piperine (50 - 400 µg/ml) and plumbagin (10 - 200 µg/ml) were analyzed as describe in section 3.8.2 for studying the linearity. Three separate calibration curves of each standard obtained on different days by plotting the peak area versus concentration. The results are shown in Table 4.10.

Table 4.10 Parameters of quantitive evaluation for piperine and plumbagin

Parameter	Piperine	Plumbagin
Linear range (µg/ml)	50 - 400	10 - 200
Equation	$Y = 23035X - 102552$[a]	$Y = 35887X - 96639$[a]
Linearity (r^2)	1	0.9998
LOD (µg/ml)[b]	0.80	0.22
LOQ (µg/ml)[c]	2.66	0.75

[a] Y=AX +B, where Y is peak area, X is the concentration of the analyzed sample.

[b] Limit of detection (LOD): signal to noise ratio = 3.

[c] Limit of quantitation (LOQ): signal to noise ratio = 10.

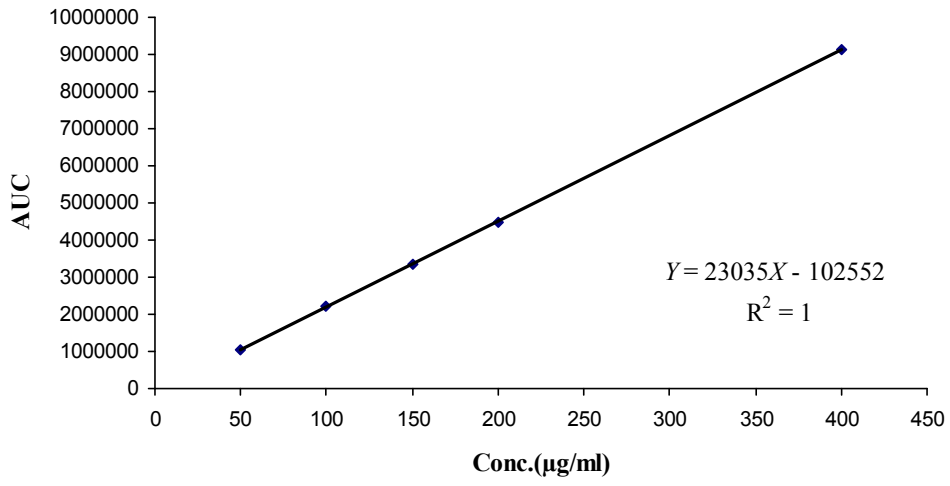


Figure 4.16 Calibration curve of standard piperine, concentrations range from 50-400 µg/ml.

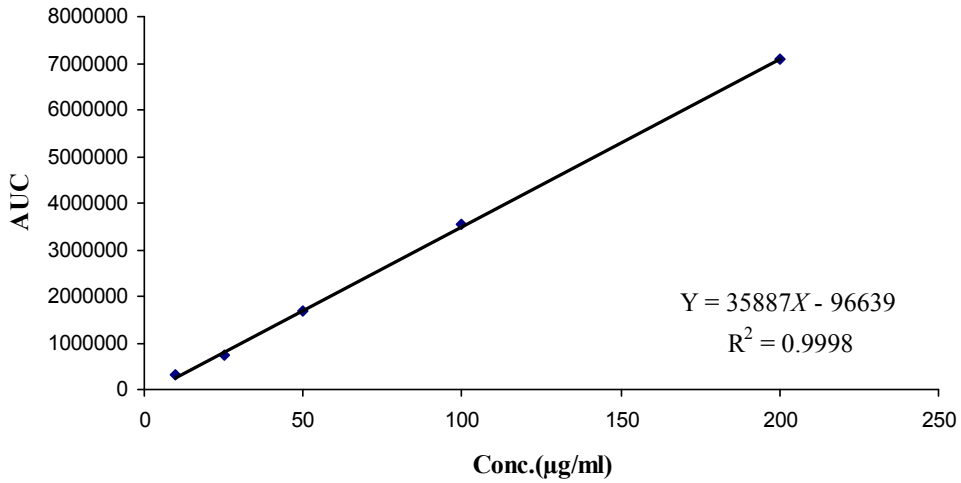


Figure 4.17 Calibration curve of standard plumbagin, concentrations range from 10-200 µg/ml.

The Results (Table 4.10) exhibited that the calibration curve of standard piperine and plumbagin were found to be linear when evaluated by linear regression analysis. The linear equation and correlation coefficient (r^2) of piperine ($Y = 23035X - 102552$, $r^2 = 1$) and plumbagin ($Y = 35887X - 96639$, $r^2 = 0.9998$) were obtained.

The limit of detection (LOD) represents the lowest concentration of piperine and plumbagin that can be detected by the instrument and the analytical method, whereas the limit of quantitation (LOQ) represents the lowest concentration of piperine and plumbagin that can be determined with acceptable precision and accuracy by the instrument and method. The results of LOD and LOQ analysis of piperine (LOD = 0.80 and LOQ = 2.66 µg/ml) and plumbagin (LOD = 0.22 and LOQ = 0.75 µg/ml) indicated that the analytical method for the quantitation of piperine and plumbagin of ethanolic extract of Benjakul preparation exhibited good sensitivity.

4.7.3 Precision validation

Both the intra- and inter-day precisions of the analytical method were studied, which obtained by triplicate analyses in a day and per day over three days, respectively. The results showed in Table 4.11 and Table 4.12.

Table 4.11 Validation of precision of the analytical method for piperine

Theoretical concentration (µg/ml)	Intra-day[a] (*n* = 3)		Inter-day[b] (*n*=9)	
	Measured concentration (µg/ml)	CV(%)[c]	Measured concentration (µg/ml)	CV(%)[c]
50	51.03 ± 0.59	1.15	50.69 ± 1.00	1.97
100	100.53 ± 0.31	0.31	100.14 ± 0.60	0.60
200	200.57 ± 1.31	0.65	199.23 ± 2.09	1.05

[a] All values are mean±SD as obtained by triplicate analyses in a day. [b] All values are mean±SD, obtained by triplicate analyses per day over 3 days.

[c] Coefficient of variation = SD/mean×100%.

Table 4.12 Validation of precision of the analytical method for plumbagin

Theoretical concentration (µg/ml)	Intra-day[a] (*n* = 3)		Inter-day[b] (*n*=9)	
	Measured concentration (µg/ml)	CV(%)[c]	Measured concentration (µg/ml)	CV(%)[c]
25	23.46 ± 0.16	0.70	23.38 ± 0.18	0.76
50	49.95 ± 0.60	1.19	49.69 ± 0.58	1.16
100	100.22 ± 1.42	1.42	100.21 ± 1.23	1.23

[a] All values are mean±SD as obtained by triplicate analyses in a day. [b] All values are mean±SD, obtained by triplicate analyses per day over 3 days.

[c] Coefficient of variation = SD/mean×100%.

The Results from Table 4.11 and Table 4.12 found that both intra- and inter-day precisions of piperine were higher than 99%, for which 0.31–1.15% and 0.60–1.97% of coefficient variations, respectively. For plumbagin, both intra- and inter-day precisions were higher than 93%, for which 0.70–1.42% and 0.76–1.23% of coefficient variations, respectively. The results indicated that the method for quantitation of piperine and plumbagin from the ehtanolic extract of Benjakul preparation have good precision.

4.7.4 Accuracy validation

The accuracy of the method was determined by investigating the recovery of samples of spiking standard piperine and plumbagin into ehtanolic extract of Benjakul preparation and comparing the measured value to the true value, which recoveries nears to 100% indicating a good accuracy of this method obtained. The results showed in Table 4.13 and Table 4.14.

Table 4.13 Validation of the accuracy of the analytical method for piperine

Spiked level (µg/ml)	Recovery (%)[a]			Mean (%)	CV (%)[b]
	1	2	3		
50	93.39 ± 0.62	93.29 ± 0.43	94.36 ± 0.67	93.68	0.63
100	95.52 ± 0.84	95.97 ± 0.11	96.59 ± 0.17	96.02	0.56
200	97.15 ± 0.05	97.22 ± 0.03	97.52 ± 0.07	97.30	0.20

[a] All values are mean±SD as obtained by triplicate analyses.

[b] Coefficient of variation = SD/mean×100%

Table 4.14 Validation of the accuracy of the analytical method for plumbagin

Spiked level (µg/ml)	Recovery (%)[a]			Mean (%)	CV (%)[b]
	1	2	3		
25	97.93 ± 0.95	96.36 ± 0.82	97.75 ± 1.12	97.35	0.89
50	100.52 ± 1.28	99.80 ± 1.97	102.44 ± 0.92	100.92	1.36
100	96.78 ± 1.16	98.92 ± 0.89	99.18 ± 1.20	98.30	1.34

[a] All values are mean±SD as obtained by triplicate analyses.

[b] Coefficient of variation = SD/mean×100%.

The Results from Table 4.13 and Table 4.14 found that piperine and plumbagin have good recoveries, for which ranging from 93.68 to 100.92%, with 0.20-1.36% of coefficient variations. It demonstrates that the analytical method has good accuracy.

4.8 Stability of ethanolic extract of Benjakul preparation

The stability of piperine and plumbagin in the ethanolic extracts of Benjakul preparation were evaluated as describe in section 3.9 and determined contents with method in section 3.7. The results of stability testing are shown in Table 4.15 and Figure 4.18.

Table 4.15 Plumbagin and piperine contents of Benjakul extraxt after stored under accelerated condition (45 °C, 75% RH)

Day	Plumbagin content (mg/g)[a]	Piperine content (mg/g)[b]
0	2.46 ± 0.02	47.61 ± 0.42
7	2.38 ± 0.02**	47.81 ± 0.25*
15	2.26 ± 0.02**	47.53 ± 0.09**
30	1.96 ± 0.02**	46.44 ± 0.16*
60	1.38 ± 0.00**	44.38 ± 0.05**
90	1.12 ± 0.02**	44.32 ± 0.02**
120	0.71 ± 0.01**	45.03 ± 0.10**

[a] All data are calculated as the standard linear equation: $Y = 35994X - 92792$, $r^2 = 0.9997$, where Y is peak area, X is the concentration of the analyzed sample.

[b] All data are calculated as the standard linear equation: $Y = 22878X - 95581$, $r^2 = 0.9999$, where Y is peak area, X is the concentration of the analyzed sample.

[c] All data are mean±SEM as obtained by triplicate analyses.

The comparison of plumbagin and piperine contents at various time with day 0 showed significantly by * p<0.05, **p<0.0001

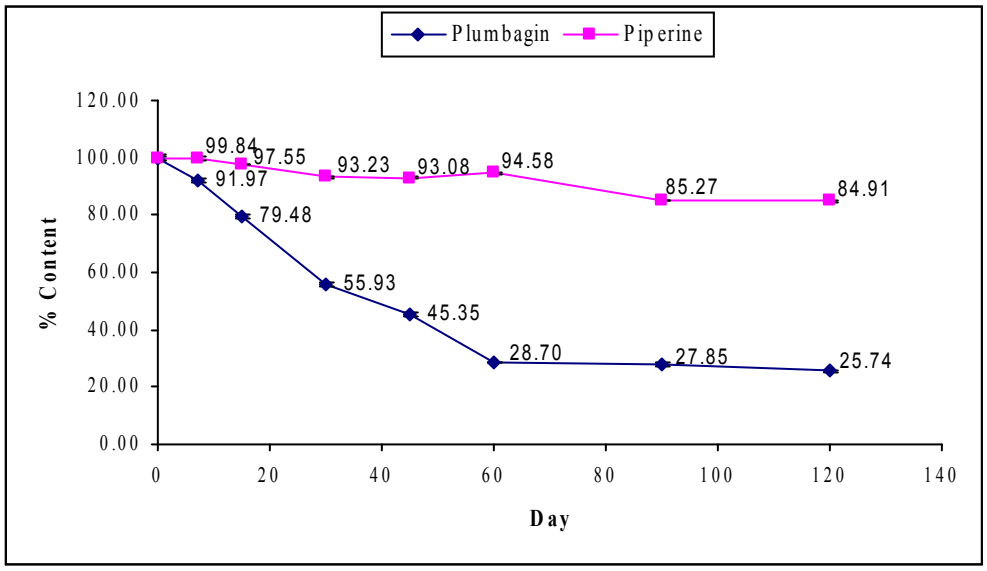


Figure 4.18 The stability of piperine and plumbagin (% content) in the ehtanolic extract of Benjakul preparation under accelerated condition (45 ± 2°C with 75 ± 5% RH) .

The results (Table 4.15 and Figure 4.18) found that the amount of piperine was a bit reduced from 47.61 mg/g (100%) at day 0 to 45.03 mg/g (84.91%) at day 120 but plumbagin was so quickly reduced. At day 0, the amount of plumbagin was 2.46 mg/g (100%) and reduced to 0.71 mg/g (25.74%) after day 120. It demonstrates that plumbagin is unstable.